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Expression of toll-like receptor (TLR)-2 and TLR4 in monocytes following stimulations by genital secretions of HIV infected and uninfected women with symptomatic vulvo-vaginal candidiasis

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Vulvo-vaginal candidiasis (VVC) is a common condition in human immunodeficiency virus (HIV)-infected women. Toll-like receptor (TLR) 2 and TLR4 are key pattern-recognition receptors of the innate immune system in sensing *Candida albicans*. The aim of this study was to assess the expression of TLR2 and TLR4 signaling pathways in HIV-infected and uninfected women with VVC. Cervico-vaginal fluids (CVF) were obtained from 7 HIV infected and 11 HIV uninfected clinic attendees in KwaZulu-Natal between June, 2011 and December, 2011. VVC was diagnosed clinically and confirmed by Gram stain and culture of genital samples. Monocytes were isolated from a healthy adult volunteer, pre-incubated with anti-TLR2, anti-TLR4 and a combination of anti-TLR2/anti-TLR4 monoclonal antibodies. Monocytes were then stimulated by CVF. Levels of cytokines were measured by Luminex® multiplex immunoassays. Compared with baseline concentrations, stimulation with CVF of HIV+VVC+ women post-TLR2 blockage increased IL-6, IL-10 and IL-13 production by 165.5, 162.5 and 106.7%, respectively. Using paired T-tests, there was a significant difference in the increase of the concentrations of IL-6 (*P* = 0.04), IL-10 (*P* = 0.003), and IL-13 (*P* = 0.031) when comparing stimulation by CVF of HIV+VVC+ versus stimulation by CVF of HIV-VVC+ patients. There was a linear correlation between genital HIV RNA loads and mean level production of IL-6 (r = 0.722; R^2 = 0.679; *P* = 0.067) as well as IL-8 (r = 0.910; R^2 = 0.833; *P* 0.004). Findings suggest potential roles of TLR2 in the pathogenesis of VVC among HIV-infected women.

Keywords: Symptomatic VVC, HIV, TLR2 and TLR4.

INTRODUCTION

In human immunodeficiency virus (HIV) infected women, symptomatic vulvo-vaginal candidiasis (VVC) is

seen to be frequent and less effectively responsive to conventional anti-fungal therapy. Reasons are not well understood. *Candida albicans* has been reported as the cause of VVC in 85 to 95% of cases (Sobel, 2007). The cell wall of *C. albicans* is composed of pathogen-associated molecular patterns (PAMPs), especially polysaccharides like chitin, 1,3-β-glucans and 1,6-β-glucans and proteins that are heavily mannosylated with mannan side-chains.

Pathogen recognition receptors (PRRs), such as the toll-like receptors (TLRs) and C-type lectins (CLRs) on the surface of antigen presenting cells (APCs) are able to recognize PAMPs. Studies have shown that TLR2 recognizes phospholipomannans; TLR4 recognizes O-linked mannans and macrophage mannose receptor (MMR) recognizes N-linked mannans (Jouault et al., 2003). Whilst the CLR dectin-1 recognizes β-glucan, CLR dectin-2 recognizes mannose residues (McGreal et al., 2006; Brown and Gordon, 2001).

Immune cell populations involved in recognition of *C. albicans* during the innate immune response include monocytes, macrophages and neutrophils. Dendritic cells are crucial for processing of and antigen presentation to T cells, and therefore for activation of specific immunity. This recognition of *C.albicans* by immune cells is done mainly through TLRs. The latter are involved in inflammatory responses induced by *C. albicans*, of which TLR2 and TLR4 are the most studied (Jouault et al., 2003). They are expressed by monocytes, macrophages, dendritic cells, neutrophils, CD4+ T cells and epithelial cells (Weindl et al., 2007). Studies have shown that the activation of TLR2 signal pathways in these antigen-presenting cells (APCs) by ligation of *C. albicans* cell-wall components such as phospholipomannan leads to the production of cytokines that are able to induce a Th2 cellular response (Weindl et al., 2007; Weis et al., 1998; Bellocchio et al., 2004; Miyazato et al., 2009). Hence, blocking TLR2 with a TLR2-specific antibody before stimulation of monocytes by *C. albicans* was shown to result in diminished release of Th2-associated cytokines (van de Veerdonk et al., 2008). In contrary, the activation of TLR4 signal pathways during candidiasis will result in the production of cytokines able to induce a Th1 cellular response. Mannans of *C. albicans* are recognized by TLR4 leading to the production of pro-inflammatory cytokines (Roeder et al., 2004).

It is however unclear whether immune changes observed at vaginal mucosal surfaces of HIV infected women interfere with the pattern recognition process of *C. albicans* by innate immune cells. Hence, the aim of this study was to assess the expression of TLR2 and TLR4 on monocytes following stimulations by genital secretions of HIV infected and uninfected women presented with symptomatic VVC. We hypothesized that HIV infection alters TLR2 (but not TLR4) dependent responses to *Candida* antigens by monocytes, resulting in symptomatic VVC.

MATERIALS AND METHODS

Study participants

A total of 18 women (7 HIV-infected and 11 HIV-uninfected), aged ≥18 years, all black Africans, attending Umlazi D clinic, a primary healthcare facility in KwaZulu-Natal between June, 2011 and December, 2011, were consecutively enrolled by informed consent. Patients aged < 18 years as well as those menstruating or having visible blood contamination of genital samples were excluded. All patients presented initially with signs and symptoms suggestive of lower genital tract infections (LGTIs) and were thereafter screened for the presence of LGTIs caused by *Chlamydia trachomatis*, *Neisseria gonorrhoeae, Mycoplama genitalium, Trichomonas vaginalis*, herpes simplex virus type 2 and bacterial vaginosis as described elsewhere (Zimba et al., 2011; Apalata et al., 2009). The selected 18 participants were retained in the study because they were free from the aetiological agents causing LGTIs. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Ref. BE 224/11).

Diagnostic criteria of symptomatic vulvo-vaginal candidiasis

Vaginal swab (Becton Dickinson) taken from the anterior fornix was directly plated onto Sabouraud Dextrose agar with chloramphenicol (BBL™ Becton Dickinson) and incubated at 29°C, 48 h to estimate the relative vaginal fungal burden. The numbers of yeast colonies were recorded as the number of colonies per plate (Sherrard et al., 2011). Cases of symptomatic VVC were defined according to clinical and laboratory criteria as described by the 2011 European (IUSTI/WHO) guideline on the management of vaginal discharge (evidence level III, recommendation grade B) (Sherrard et al., 2011).

Isolation of monocytes from peripheral human blood

Using Histopaque® 1077 and Histopaque® 1119 (Sigma-Aldrich®) per manufacturer's instructions, we isolated neutrophils and monocytes from fresh human blood collected from a healthy donor (neutrophils were used for other experiments not discussed here) (Rubin-Bejerano et al., 2003). After centrifugation and different wash steps, the peripheral blood mononuclear cells (PBMCs) appeared as a dense white band above the Histopaque® 1077 and granulocyte layer. This was removed with a 5 ml plastic pipette. Monocytes were separated from lymphocytes on the basis of their differential adherence to plastic (Rubin-Bejerano et al., 2003). The

cells were finally resuspended into 2 ml RPMI-1640 medium supplemented with D-glutamate (HiMedia Laboratories, Mumbai, India). The cell count was done using the dye exclusion test. Briefly, a total of 90 µl of isotonic phosphate buffered saline (PBS; Oxoid Limited Basingstoke, UK) (pH = 6.9) and 10 µl of monocytes was mixed and added to 100 µl of Trypan blue solution, 0.4% (Gibco®). The number of cells was counted with a haemocytometer under an inverted microscope and adjusted to 1×10^6 cells/ml.

Collection of and stimulation of monocytes with cervico-vaginal fluids (CVF)

A vaginal tampon (8 Ks), Tampax Regular® (Compak) was inserted into the vagina, left *in situ* for 3 min and then placed into a sterile container containing 10 ml of phosphate buffered saline (PBS; Oxoid Limited Basingstoke, UK) (pH = 6.9). Vaginal fluid was expressed using an autoclaved wooden tongue depressor and filtered through a 0.22 µm Costar Spin-X cellulose acetate filter membranes (Sigma).

In testing the roles of TLR 2 and TLR 4, monocytes were pre-incubated (1 h at 37°C) separately with anti-TLR2 (Abcam®) and anti-TLR4 (Abcam®) specific monoclonal antibodies before stimulation with CVF or sterile normal saline (negative control) into 96 wells tissue culture plates. A total of 500 µl of 1×10^6 monocytes/ml were pre-incubated with anti-TLR2 (50 µl). Another 500 µl of 1×10^6 monocytes/ml were pre-incubated with anti-TLR 4 (50 µl). Another 500 µl of 1×10^6 monocytes/ml were pre-incubated with a mixture of 50 µl of anti-TLR 2 and 50 µl of anti-TLR 4 antibodies. We also used 500 µl of 1×10^6 monocytes/ml pre-incubated with 50 µl of sterile normal saline (no anti-TLR antibodies) as controls. At the end of the pre-incubation period, 50 µl of 1×10^6 monocytes/ml were mixed with 50 µl of CVF obtained from HIV infected and HIV uninfected women diagnosed with symptomatic VVC. The mixture was incubated into 96 wells plate at 37°C and supernatant was collected after 4 and 24 h following stimulation in order to measure cytokines and chemokines.

Measurement of cytokines/chemokines

Concentrations (in pg/ml) of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β, TNF-α, TGF-β1, TGF-β2 and TGF-β3 were measured by a multiplex microbead system (Invitrogen, UK) using a Luminex platform. Multiplex cytokine fluorescent bead-based immunoassays were performed using two different commercially available multiplex luminex kits: Bio-plex pro human cytokine 17-plex assay and Bio-plex pro TGF-β 3-plex assay (Bio-Rad Laboratories, Inc., Parkwood). The assay sensitivity or limit of detection (pg/ml) was: IL-1β (0.6), IL-2 (1.6), IL-4 (0.7), IL-5 (0.6), IL-6 (2.6), IL-7 (1.1), IL-8 (1.0), IL-10 (0.3), IL-12 (3.5), IL-13 (0.7), IL-17 (3.3), G-CSF (1.7), GM-CSF (2.2), IFN-γ (6.4), MCP-1 (1.1), MIP-1β (2.4), TNF-α (6), TGF-β1, TGF-β2 and TGF-β3; and a 5 PL regression formula was used to calculate cytokine/chemokine concentrations from the standard curves (Bio-Plex Manager software, version 4). Cytokine/chemokine concentrations below the lower limit of detection were reported as the midpoint between the lowest concentrations measured for each cytokine and zero.

Statistical analysis

Data were expressed as means ± standard error of the mean (SEM) for the continuous variables and proportions (percentages) for the categorical variables. When data were normally distributed, analysis of the variance (ANOVA) was used to examine differences between groups. However, non-parametric Mann-Whitney U or Kruskal-Wallis tests were used when data were asymmetrically distributed. Multiple comparisons of means of cytokine/chemokine levels displaying significant differences in univariate analyses across the study groups were performed using Post Hoc Bonferroni pairwise tests considering a type I error rate of 0.05. For normally distributed variables, Paired T-tests were used to determine if two sets of variables were significantly different from each other. Data were analysed using SPSS® statistical software version 21.0 (SPSS Inc; Chicago, IL). All tests were two sided and a p value < 0.05 was considered as significant.

RESULTS

Of the 18 participants, symptomatic VVC was diagnosed from 7/7 (100%) of HIV infected and 6/11 (54.5%) of HIV uninfected women. Following blockage of TLR2 with an anti-TLR2 monoclonal antibody, monocytes were stimulated with CVF of HIV-VVC-, HIV-VVC+ and HIV+VVC+ women. Table 1 depicts the mean concentrations of cytokines/chemokines displaying significant differences across those 3 study groups. Bonferroni multiple comparison tests were performed for variables that showed significant univariate associations (Table 1). Of the 5 cytokines that showed significant differences across the study groups, 2 anti-inflammatory (IL-10 and IL-13) and 1 pro-inflammatory (IL-6) cytokines were confirmed by Bonferroni tests. The mean level of IL-6 was significantly higher in HIV+VVC+ group as compared to HIV-VVC+ group (P = 0.03). In addition, there were significantly higher mean levels of IL-10 (P = 0.003) and IL-13 (P = 0.019) in HIV+VVC+ group as compared to HIV-VVC- group.

Compared with baseline concentrations, stimulation with CVF of HIV+VVC+ women post-TLR2 blockage increased IL-6, IL-10 and IL-13 production by 165.5, 162.5 and 106.7%, respectively (Figure 1). However, stimulation with CVF of HIV-VVC+ women only increased IL-6, IL-10 and IL-13 by 36.8, 65.9 and 66.7%, respectively (Figure 2). Using paired T-tests, there was a significant difference in the increase of the concentrations of IL-6 (P = 0.04), IL-10 (P = 0.003) and IL-13 (P = 0.031) when comparing stimulation by CVF of HIV+VVC+ versus stimulation by CVF of HIV-VVC+ patients. Stimulation post-TLR4 blockage by CVF of HIV-VVC+ and CVF of HIV+VVC+ women did not show significant differences of the mean concentrations of all tested cytokines across the study groups. After blocking TLR2 and TLR4 simultaneously with specific monoclonal antibodies, only IL-6, IL-10 and IL-13 were significantly increased when monocytes were stimulated with CVF of HIV+VVC+ as depicted in Figure 3. Findings also showed a linear correlation between genital HIV RNA loads and mean level production of IL-6 ($r = 0.722$; $R^2 = 0.679$; P = 0.067)

Table 1. Comparison of cytokinr/chemokine mean levels across the groups post-LTR2 blockage.

Variable	Study group (mean ± SEM)			P value
	HIV- and VVC- (n=5)	HIV- and VVC+ (n=6)	HIV+ and VVC+ (n=7)	
Anti-inflamatory				
IL-10	0.02±0.001	0.42±0.12	0.69±0.12	0.001
IL-13	0.15±0.001	0.25±0.001	0.31±0.04	0.019
TGF-β2**	60±0.001	47.37±4.23	51.6±1.69	0.021
Pro-inflamatory				
IL-6	0.35±0.001	0.26±0.08	0.77±0.16	0.021
MCP-1**	1.05±0.001	0.93±0.004	1.01±0.12	0.028

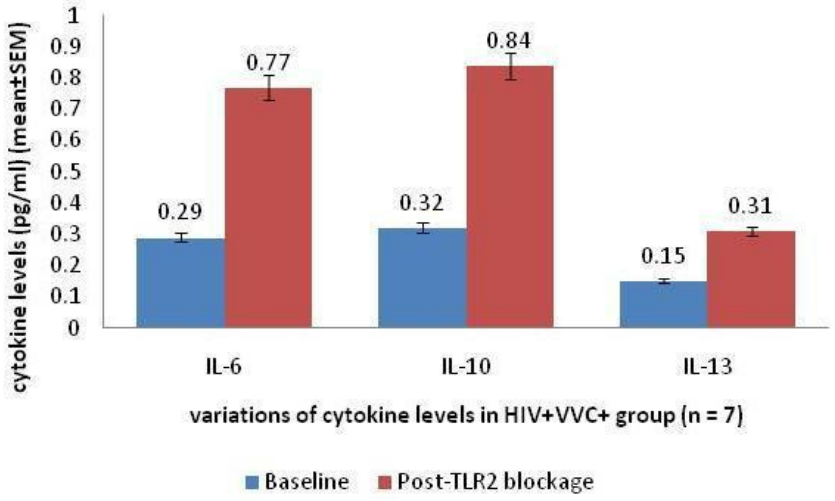

Figure 1. Variations of cytokine levels in HIV infected women.

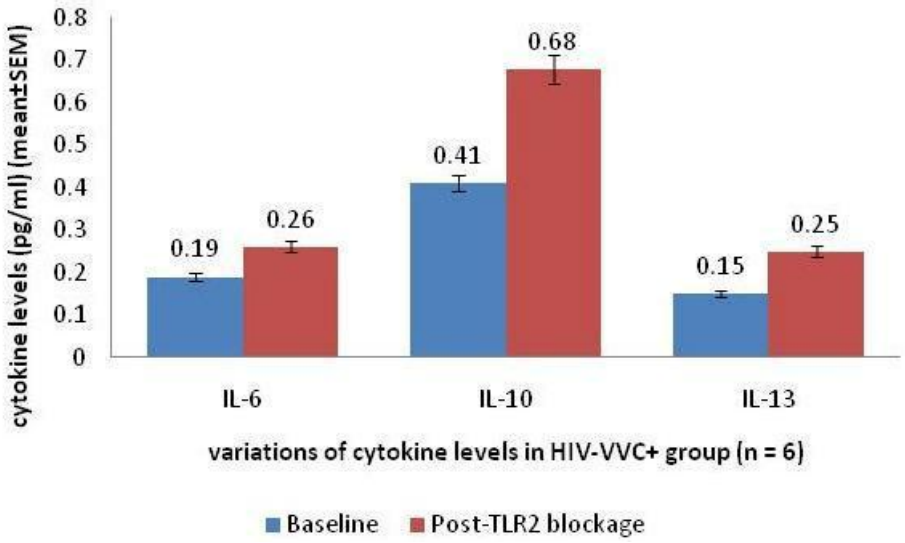

Figure 2. Variations of cytokine levels in HIV uninfected women.

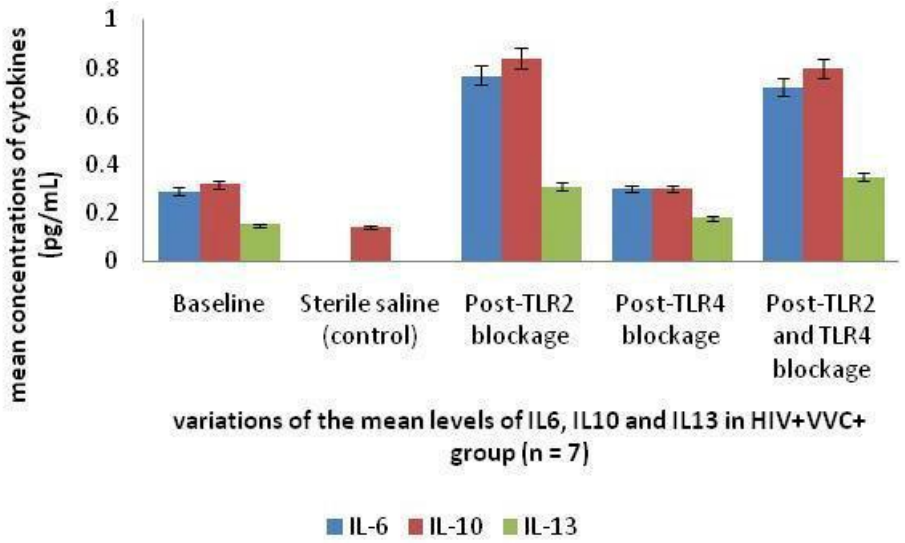

Figure 3. Post-TLR2 and TLR4 blockage cytokine levels in HIV infected women.

as well as IL-8 ($r = 0.910$; $R^2 = 0.833$; $P = 0.004$).

DISCUSSION

Findings from this study have shown that blocking TLR2 with TLR2-specific antibody followed by stimulation of monocytes with CVF of HIV-infected women who were also co-infected with symptomatic VVC resulted in higher increase in the concentrations of IL-6, IL-10 and IL-13. HIV+VVC+ patients produced unusually high levels of one inflammatory cytokine (IL-6) and 2 potent anti-inflammatory cytokines (IL-10, IL-13).

Studies in mice have shown that the development of protective anticandidal Th1 responses requires the concerted actions of several pro-inflammatory cytokines in the relative absence of inhibitory Th2 cytokines, such as IL-4 and IL-10, which inhibit development of Th1 responses (Tonnetti et al., 1995). In the present study we found that despite increased mean levels of IL6, there was a parallel excess production of IL-10 and IL-13 post-TLR2 blockage. These findings confirm that in HIV-infected patients, Th1 activation results in phagocyte-dependent immunity and might represent an important mechanism of anticandidal resistance; but subsequent Th2 reactivity, triggered by *Candida* infection would be mostly associated with the pathology (Romani et al., 1995). This can suggest that Th2 reactivity overcame the Th1 responses in HIV positive women leading to candidiasis. Thus, the Th cell dichotomy to *Candida* may have important implications particularly in contributing to the dominance of Th2 responses in cases of recurrent VVC observed among HIV positive women (Romani et al., 1995).

IL-13 is recognized for its effects on monocytes, where it upregulates class II expression, promotes IgE class switching and inhibits inflammatory cytokine production. In general, activation of TLR2 signal pathways during candidiasis should lead to the production of Th2 cytokines, thus blocking TLR2 with a TLR2-specific monoclonal antibody followed by stimulation of monocytes by *C. albicans* should result in diminished release of these Th2 cytokines. Netea et al. (2004) showed that $TLR2^{-/-}$ mice are more resistant to disseminated *Candida* infection and this is associated with increased chemotaxis and enhanced candidacidal capacity of $TLR2^{-/-}$ monocytes/macrophages. Whilst the production of pro-inflammatory cytokines can be normal, levels of anti-inflammatory cytokines are severely impaired in the $TLR2^{-/-}$ mice. The authors found that this was accompanied by a substantial decrease in the CD4+CD25+ regulatory T (Treg) cell population in $TLR2^{-/-}$mice (Netea et al., 2004). Furthermore, *in vitro* studies confirmed that enhanced survival of Treg cells was induced by TLR2 agonists; *C. albicans* induces immunosuppression through TLR2-derived signals that mediate increased anti-inflammatory cytokine (that is, IL-10) production and survival of Treg cells, playing a critical role in the pathogenesis of symptomatic VVC.

CONCLUSION

The present study demonstrated that in HIV-infected

individuals, there might be an upregulation of TLR2 during stimulation of monocytes by *Candida* spp. leading to an over production of anti-inflammatory cytokines. This might suggest an underlying role played by Th2/Treg cell populations during HIV infection.

LIMITATION

The limitation of this study is mainly the small sample size that might not allow generalization of our findings. A further study is thus warranted.

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